**The Final Report**

**on**

# Neutron Irradiation Studies Involving Newly-Developed Cholesteryl Carborane Ester Compounds

Georgia Tech Proposal No.: 02.500.507.04.97272/Coalition Agreement GCC-66

Sumitted to

**Georgia Cancer Coalition**

by

Prof. C-K Chris Wang

December, 2009

Nuclear & Radiological Engineering & Medical Physics Program
School of Mechanical Engineering
Georgia Institute of Technology

## 1.0 Introduction

The purpose of this project was to evaluate the efficacy of a newly developed $^{10}$B-containing compound - Cholesteryl carborane ester compound (or BCH) using human prostate cancer cells PC-3 for boron neutron capture therapy (NCT). The BNCT is an experimental cancer treatment modality, which includes two sequential steps: (1) a thermal neutron absorbing agent (e.g. a $^{10}$B-containing compound) is first administered into a patient, and (2) the tumor is then irradiated with thermal neutrons. The compound is designed to preferentially enter the tumor cells. During irradiation, a boron-10 atom in a tumor cell would absorb a thermal neutron and the energetic nuclear reaction products (i.e. the alpha particle and the lithium-7 ion) would subsequently kill the cell. Because the therapeutic efficacy of BNCT highly depends on the effectiveness of the compound (i.e. its ability to preferentially enter tumor cells), each new compound must first be evaluated in cell cultures and small animals before it can be used for human clinical trials.

The new $^{10}$B-containing compound (BCH) to be evaluated in this project was synthesized and provided by the University of Georgia Professor, Dr. Robert D. Lu (who now works at the FDA in Washington, D.C.). It was thought that because BCH mimics the structure of native cholesteryl esters, it may be incorporated into the low density lipoprotein (LDL). Earlier studies showed that BCH carried by liposomes resulted in an effective transfer of BCH into LDL in biological fluid and that the amount of BCH taken by the glioma cell lines (SF-767 and SF-763) was much more (up to 14 times) than that taken by the normal neuron cell line[1,2]. Another recent study showed that prostate cancer cells also express more LDL receptors than normal prostate cells do in the presence of LDL or whole serum[3]. As such, we believe that prostate cancer cells will also take up more BCH than the normal prostate cells do and that BCH has the potential to be used in a BNCT for treating human prostate cancer.

The original proposal of this project included four major tasks: (1) to set up a new cell culture laboratory, (2) to prepare liposomal formulation with BCH and to maximize the uptake of BCH by the human prostate cancer cells (PC-3), (3) to quantitatively analyze the BCH (or $^{10}$B) uptake and egress of PC-3 cells, and (4) to irradiate the BCH-loaded prostate cancer cells with thermal neutrons and then conduct the cell survival analysis (i.e. the clonogenic assay). Due to the nature of how BNCT works, the four tasks must go in sequence.

## 2.0 Work Completed

As previously reported[4], while tasks (1) and (2) were completed in 2004, tasks (3) and (4) had to be delayed due to the remodeling of thermal column facility at the University of Maryland Training Reactor (MUTR) where the experiment was proposed to be carried out. Consequently, the PI requested for no-cost extension of the project for the several years. The remodeling of

thermal column was finally completed in June 2007[5], and the proposed experiment was then pursued at MUTR (see Attachment A). Unfortunately, as of today the researchers at MUTR have not yet produced reliable results for the BCH uptake to PC-3 cells. They have found that extrusion restricts the amount of drug in the liposomal formulation due to membrane-drug interaction and have now switched to a new method of making liposomes. There are some preliminary indications that this new method will work much better.

While the project is still being continued at MUTR at a slow pace (due to lack of funding), at this junction the PI can no longer expect that tasks (3) and (4) be completed in a timely manner. As such, the PI would like to use Attachment A as the final report to conclude this project. Should tasks (3) and (4) be completed in the future and if GCC is still interested in receiving the results, the PI will be happy to submit them to GCC.

## 3.0 References


1. Peakcock, G., Ji, B., Wang, C.K., and Lu, D.R., "Cell Culture Studies of a Carborane Cholesteryl Ester with Conventional and PEG Liposomes," Drug Delivery, **10**, pp. 29-34. 2003.

2. Ji, B., Chen, W. and Lu, D. R., "Cell culture and animal studies for intracerebral delivery of borocaptate in liposomal formulation," Drug Delivery, **8**, pp. 1-5, 2001

3. Chen, Y. F. and Hughes-Fulford, M: Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator, SREBP2, Int. J. Cancer, **91**, pp. 41-45, 2001.

4. Wang, C. K., "A progress report on neutron irradiation studies involving newly-developed cholesteryl carborane ester compounds," submitted to Georgia Cancer Coalition, September, 2004.

5. E-mail from Professor Al-Sheikhly of University of Maryland, June 11, 2007. Professor Al-Sheikhly can be reached at: (301) 405-5214 and mohamad@umd.edu.

6. Wang, C. K. and Zhang, X., "A nanodosimetry-based linear-quadratic model of cell survival for mixed-LET radiations," Physics in Medicine and Biology, **51**, pp. 6087-6098, 2006.

# ATTACHMENT A

# ENHANCED BORON NEUTRON CAPTURE THERAPY FOR THE TREATMENT OF PROSTATE CANCER USING A NEW BORON-CONTAINING CHOLESTERYL ESTER COMPOUND

By

Ian Alexander Gifford

Proposal submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy
2008

ABSTRACT

Title of Document: Enhanced boron neutron capture therapy for the treatment of prostate cancer using a new boron-containing cholesteryl ester compound

Ian A. Gifford, Bioengineering

Directed By: Mohamad Al-Sheikhly, Professor, Materials Science and Engineering

The use of a novel boron-containing cholesteryl carborane ester compound (BCH) with boron neutron capture therapy (BNCT) for the treatment of prostate cancer is under study. Specifically, (1) the cellular uptake of this compound and (2) the efficiency of thermal neutron irradiation of cancerous prostate cells loaded with this compound are under investigation. BCH is incorporated into a liposome formulation and analyzed with multi-angle light scattering to determine the size of the liposomes. Boron-containing liposomes are then added to the cell growth medium of normal human prostate cells (RWPE-1) and cancer human prostate cells (PC-3), followed by analysis of BCH uptake and release with high performance liquid chromatography. Variations in liposome BCH concentration and incubation time will determine the conditions for optimal therapeutic ratio. Thermal neutron irradiation will be delivered through a newly designed plug within the 250 kW University of Maryland research reactor thermal column which allows environmental control throughout the irradiation. Beam characterization and neutron spectrum unfolding will be completed to determine the dose rates within the sample chamber. Cell survival fractions of normal and cancer human prostate cells will reveal the efficacy of this targeted radiotherapy. Results from this project will assist in determining the feasibility of using these new ester compounds for prostate cancer treatment.

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## I. Introduction

According to the American Cancer Society, prostate cancer is the most frequently diagnosed cancer and the leading cause of cancer death in men. It is estimated that 218,890 new cases of prostate cancer occurred in the US during 2007, with 27,050 cases resulting in death.[1] Because of this, the development of new treatment options involving targeted drug delivery which may improve the efficacy of prostate cancer treatment is essential.

The purpose of targeted drug delivery is to deliver more anti-cancer drug to cancer cells than to normal cells, resulting in fewer side effects for the cancer patient. One of the fundamental approaches is to design new anti-cancer compounds that capitalize on the differences between cancer and normal cells. It has been reported that prostate cancer cells, like many other types of cancer cells, have high expression of low-density lipoprotein (LDL) receptors in order to obtain more cholesterol to accelerate their characteristic abnormal growth.[2] Therefore, targeted drug delivery can be accomplished by synthesizing cholesterol-based anti-cancer compounds specific to the LDL receptors. With this in mind, a new anti-cancer cholesteryl carborane ester compound (BCH) has been developed for testing. This compound is designed to mimic the native cholesteryl esters in the human body and thus follow the same pathway to be selectively taken up by cancer cells through the elevated expression of LDL receptors in the cancer cell membrane.[3,4]

This research will help to evaluate the ability of thermal neutron irradiation on prostate cancer cells containing the new carborane compound, BCH, to treat prostate cancer. This compound has the potential to selectively target prostate cancer cells for boron neutron capture therapy (BNCT). Specifically, (1) comparison between the cellular uptake and release of the new cholesteryl carborane compound in human normal and cancer cells and (2) examination of the efficacy of BNCT through neutron irradiation on human normal and cancer prostate cells loaded with BCH. The results of this project will help evaluate the feasibility of the new class of anti-cancer compounds for prostate cancer treatment.

## II. Specific Aims

The objective of this research project is to investigate the efficacy of a new boron-containing cholesteryl ester compound, BCH, as a targeting compound for BNCT of prostate cancer. It is proposed that this compound will successfully target human prostate cancer cells and result in the radiation induced killing of cancer cells following the delivery of a dose of thermal neutrons. In order to adequately determine this, the ability to prepare boron-containing liposomes, cellular uptake and release of the BCH, and cell survival following thermal irradiation must be studied.

First, incorporation of BCH into a liposomal formulation and subsequent liposome size distribution analysis must be completed. To accomplish this task, liposomes with varying concentrations of BCH will be prepared by the thin film hydration method and extruded through membranes of various sizes. Light scattering analysis will provide information regarding the size distribution of the liposomes as a function of BCH concentration and extrusion membrane size.

Second, the uptake and release kinetics of the BCH by the normal and cancer human prostate cells will the determined. It is anticipated that the cholesterol seeking cancer cells expressing high levels of LDL receptors on their cell membranes will endocytose more BCH than the normal cells. High performance liquid chromatography will be utilized to determine the cellular concentration of BCH in each cell population following various incubation times. This data will indicate the time when peak BCH concentration is reached in the cancer cells, as well as providing release kinetics to ensure the BCH concentration remains high throughout the cell irradiation.

Third, cell survival assays must be performed following thermal neutron irradiation of the cells. Cells containing boron will encounter high LET reactions resulting in fatal cell damage, while cells without BCH should be able to recover from the relatively low dose of thermal neutrons. By irradiating when there is peak BCH concentration in the cancer cells, the potential for a fatal double stranded DNA break is at its highest.

Upon completion of these tasks, a detailed assessment of the efficacy as a potential BNCT drug for prostate cancer will be made.

## III. Technical Background

*Boron Neutron Capture Therapy*

Boron neutron capture therapy (BNCT) is a binary radiation cancer treatment with potentially significant therapeutic advantages. The treatment is based on specific cell killing by neutron irradiation of cancer cells that have been loaded with a boron-containing compound. The nuclear reaction between a thermal neutron (<0.025 eV) and a non-radioactive $^{10}B$ atom produces a high linear energy transfer (LET) $\alpha$ particle and $^{7}Li$ nuclei.

$$^{10}B + {}^{1}n \rightarrow {}^{7}Li + {}^{4}He + 2.4\ MeV \qquad (1)$$

These high LET particles impart their energies in a relatively short range, approximately 9 and 5 μm respectively.[5] Because this distance is on the same scale as a typical cell diameter, the particles are capable of fatally damaging cells containing the $^{10}B$, while leaving neighboring cells unharmed.[6-8] The 1.7 MeV $\alpha$ particles that result from this capture reaction have an average LET of 200 keV/μm, therefore capable of killing the cells when only a few particles deposit their energy.[9] Figure 1 provides a schematic for this reaction.

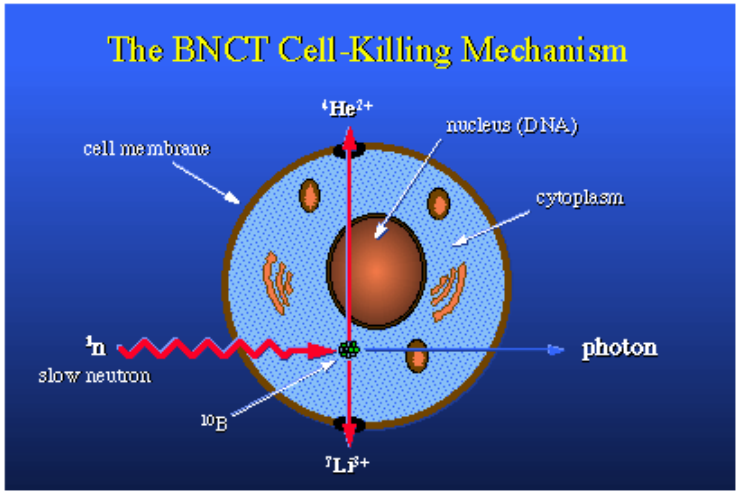


**FIGURE 1.** BNCT cell killing mechanism.

It is important to note that hydrogen and nitrogen found within the normal tissue can also participate in capture reactions to produce gamma-rays and protons.

$$^{1}H + {}^{1}n \rightarrow {}^{2}H + \gamma\, 2.23\ MeV \qquad (2)$$

$$^{14}N + {}^{1}n \rightarrow {}^{14}C + {}^{1}p\ 0.63\ MeV \qquad (3)$$

However, the thermal neutron cross sections of these atoms are very small (0.332 and 1.75 barns, respectively) compared to that of $^{10}B$ (3,838 barns) and will only deliver a small percentage of the overall dose.

There are several advantages of BNCT for targeted treatment. (1) Because the boron-containing compounds themselves are neither required to be cytotoxic nor to release the anti-cancer units in the cells, boron conjugates can be designed solely for targeted delivery purposes. (2) Radiation can be applied in a time window (radiation window) when the concentration ratio of boron-containing compounds in the cancer cells versus the surrounding normal tissue reaches a maximum. (3) BNCT is particularly suitable for prostate cancer treatment because the physiological location of the prostate is distant from many vital organs, and can thus be targeted with a direct neutron beam.[5,7,10] In addition, the prostate lays 1.0-1.5 cm from the body surface and thus moderates an epithermal neutron beam (0.025-1 eV) to the appropriate energy as the neutrons pass through tissue.[11]

Clinical trials for BNCT of glioblastoma multiforme (GBM) began in the 1950s at the Brookhaven National Laboratory (BNL) and the MIT reactor (MITR) and were stopped in 1961 due to disappointing outcomes.[12,13] Analysis of the data showed that biodistribution studies and improved characterization of radiation sources were key factors that needed to be addressed. Studies began again in Japan in 1967 using BPA as the boron-containing agent in combination with tumor “debulking”. Thermal neutron beams were utilized that required the opening of the skull to increase neutron penetration and limit exposure to the skin.[14] While failing to provide an increase in patient survival times, the results were promising compared to previous reports and trials began once

again in the USA and Europe in 1994. The key difference in these trials was the use of epithermal neutron beams that would provide deeper tissue penetration while avoiding opening of the skull as performed in the Japanese trials. The trials at BNL and MITR have been completed and produced results comparable to conventional photon therapies.[15] Trials from Sweden and Finland have shown considerable increases in survival times compared to trials in the USA, although direct comparisons are difficult due to variations in therapy plans. Other clinical trials are underway for BNCT treatment of melanomas, head and neck tumors, and colon adenocarcinoma. A complete review of these trials is beyond the scope of this proposal and can be found elsewhere.[6,16]

*Design and Synthesis of Boron-containing Compounds*

The overall success of BNCT lies in the ability to synthesize boron-containing compounds that satisfy the following requirements: (1) selective targeting of cancer versus normal cells, (2) cellular concentrations of ~$10^9$ $^{10}$B atoms/cell or ~20-35 μg/g tumor, (3) tumor-to-normal tissue differential in the 3-5 range, (4) persistent cellular concentration of $^{10}$B throughout the irradiation procedure, and (5) low toxicity levels at doses required to attain adequate cellular concentrations.[7,17-19]

Currently, there are three boron-containing that are approved for clinical trials by the US Food and Drug Administration (FDA) with Investigational New Drug classification. The first, borocaptate sodium $Na_2$[*closo*-$B_{12}H_{11}SH$] (BSH), is a carborane derivative containing ten $^{10}$B atoms per molecule that has been used in BNCT treatment of GBM and its biodistribution in animals has been studied.[20-24] The second, 4-dihydroxyborylphenyl-alanine (BPA), is an amino acid containing one $^{10}$B atom per molecule that has been used to treat patients suffering from malignant melanoma and GBM, with similar studies in animal biodistribution.[25-30] The third, polyhedral borane dianion [*closo*-$B_{10}H_{10}$]$^{2-}$ (GB-10), has been studied for its ability to treat GBM.[31,32] However, each of these compounds has its own limitations in selective tumor targeting and retention leading to interest in the development of new compounds with the ability to better satisfy the criteria previously described. A more complete discussion of these approved compounds can be found elsewhere.[5,33] In addition to these FDA approved

drugs, many other compounds have been suggested as BNCT candidates including, but not limited to: boronated porphyrins, boronated nucleosides and nucleotides, monoclonal antibodies, and growth factors.[5,7]

A promising new cholesteryl carborane ester compound (BCH) has been synthesized that mimics the structure of native cholesteryl ester but contains a carborane cage as the source of boron for BNCT.[3,4] This compound will be evaluated in this research project for targeted therapy of prostate cancer. The structure of BCH is shown in Figure 2.

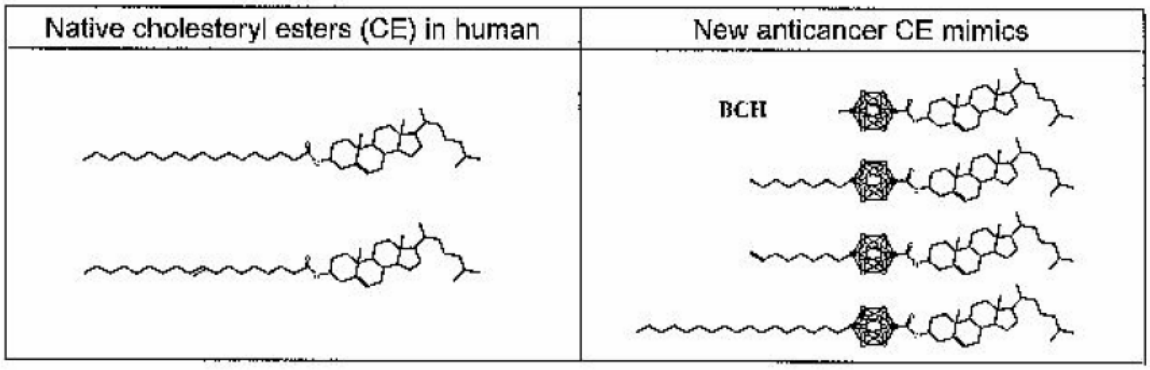


**FIGURE 2.** Cholesteryl carborane esters in comparison with native cholesteryl esters.

Similar to BSH, a boron cage is attached to the end of the molecule which contains ten $^{10}B$ atoms. In addition to this, the *para*-carborane cage increases the lipophilicity of the molecule which may improve incorporation of the molecule into the low density lipoproteins (LDL).[34-36] Due to the high hydrophobicity of the BCH molecule, incorporation into a liposomal formulation is required for delivery into a biological system.[4] Studies involving BCH uptake of normal neuron cells and brain glioma tumor cells show the ability of this anti-cancer agent to preferentially target cancer cells.[37]

| Cell line | Cellular BCH uptake (□g boron/g cell) |
|---|---|
| Normal neuron cell, HCN-1a | 19.9 ± 2.9 |
| Brain glioma tumor cell, SF-763 | 240.1 ± 22.3 |
| Brain glioma tumor cell, SF-767 | 283.4 ± 48.1 |

**TABLE 1.** Cellular uptake of BCH by different cell lines.

*Targeted Drug Delivery*

The true success of BNCT rests in the ability to specifically target cancer cells for delivery of boron while limiting the uptake of boron by normal cells. Methods to address this issue include synthesis of individually targeting boron-containing compounds, conjugation of boron-containing compounds to macromolecules, and encapsulation of the compounds in microcapsules.[5,7] However, complications have arisen in each case which make them less than ideal for adequate delivery of $^{10}B$ to the targeted cells.

Another method for targeted delivery of $^{10}B$ to cancer cells is based on the over-expression of low-density lipoprotein (LDL) receptors by a variety of cancer cells.[38,39] Specifically, it has been reported that human prostate cancer cells (PC-3) lack the feedback regulation of LDL receptors seen in normal human prostate cells.[2] LDL is a 20-25 nm particle consisting of a lipid monolayer containing apolipoprotein that binds to LDL receptors, surrounding approximately 1,500 cholesteryl esters within its hydrophobic core. LDL is the primary carrier of cholesterol in the blood stream and accounts for up to 90% of cholesterol found in the cell, the rest resulting from *de novo* synthesis.[40]

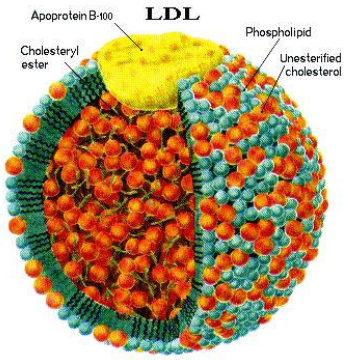


**FIGURE 3**. Low-density lipoprotein.

LDL receptors are more active in rapidly dividing cancer cells because the formation of new cell membranes requires high levels of cholesterol. The pathway for cellular uptake of LDL is termed receptor-mediated endocytosis and relies on cellular synthesis of

transmembrane LDL receptors that are expressed on the cell surface. Once LDL binds to the receptor, the particle is endocytosed into the cell and transported to the lysosomes, where the LDL particles are hydrolyzed to form free cholesterol that can be used by the cells. In normally functioning cells, feedback regulation prevents expression of LDL receptors on the cell surface once enough cholesterol has been accumulated within the cell. In cancer cells however, this feedback is not present and cells will continue to endcytose LDL. Therefore, successfully incorporating BCH into LDL will provide a means of not only preferentially targeting cancer cells but also facilitating intracellular delivery of the boron.

In order to introduce BCH into the LDL, the extremely hydrophobic compound must be incorporated into multi-lamellar vesicles (MLVs). The MLV liposomes contain a mixture of DL-α-dipalmitoylphosphatidylcholine, cholesterol, and BCH. As described later in detail, the compounds are mixed and dried to form a thin film which is then hydrated and agitated to form the MLVs.

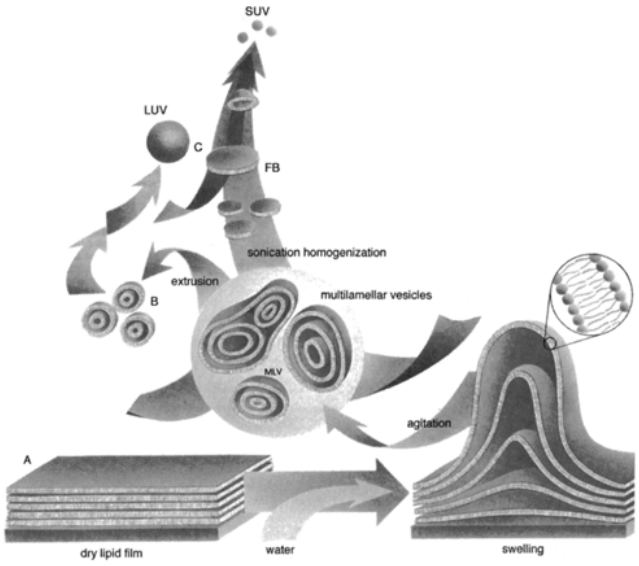


**FIGURE 4.** Mechanism of vesicle formation (Avanti Polar Lipids, Inc.).

Following extrusion through a membrane, the MLVs mix with LDL in the cell medium. The liposomes provide a means of solubilizing the BCH and facilitate interaction with the LDL. Liposomes are able to interact with LDL through mechanisms of fusion, lipid

transfer, and lipid exchange because they are both lipid based particles, creating an efficient means of loading hydrophobic drugs into LDL.[35,37,41,42] The uptake of BCH through the LDL pathway has been demonstrated in glioma cell lines using anit-LDL receptor antibodies to block the endocytosis of BCH, resulting in a significant decrease in uptake of BCH.[37]

| Cell line | Cellular BCH uptake (□g boron/g cell) |
|---|---|
| Brain glioma tumor cell, SF-767 | 283.4 ± 48.1 |
| Brain glioma tumor cell, SF-767 | 69.3 ± 9.6 * |

**TABLE 2.** BCH uptake through the lipoprotein endocytosis pathway.
* Lipoprotein deficient serum used in the culture medium.

*Thermal Neutron Irradiation*

An area of importance for the success of BNCT is the availability of a thermal neutron source. There is great interest in the development of accelerator based neutron sources for use in hospital settings, however to date there is no such setup. Development of this type of neutron source would be a landmark step in the clinical application of BNCT and is discussed in great detail elsewhere.[6,7,43,44] Currently, nuclear reactors are the only viable source of neutrons for BNCT. Within the reactor core, neutrons are born from $^{235}U$ fission with energy ranging from fast (>1 eV), to epithermal (0.025-1 eV), to thermal (<0.025 eV). In order to improve the probability of interaction between neutrons and boron atoms within the cell, neutrons must be thermalized prior to capture by $^{10}B$. This occurs by interaction with a moderator, causing neutrons to lose energy through collisions with other atoms. For clinical therapy, thermal neutrons that are incident upon the patient do not carry enough energy to penetrate the tissue to the tumor depth. To resolve this problem, clinical sites are currently investigating the use of epithermal neutron beams that are moderated as they penetrate tissue, becoming thermal upon interaction with the tumor.[45,46] Epithermal neutron beams are being utilized for BNCT therapy at reactors within the US at BNL and the MITR. In addition to this, reactors in The Netherlands, Finland, Sweden, the Czech Republic, and Japan are modifying reactors for research involving BNCT.[6] However, for research in an *in vitro* setting the

development of thermal neutron beams is of significant importance. Clearly, monoenergetic neutron beams are unrealistic and will always be contaminated with higher energy neutrons and gamma rays which lead to non-specific cell killing; but by increasing shielding and beam moderation a higher proportion of incident radiation is delivered by thermal neutrons.

The 250 kW TRIGA Maryland University Training Reactor (MUTR) will be used as the thermal neutron irradiation source. The core of the MUTR contains 93 fuel elements with UZrH fuel enriched to <20% and three borated carbide control rods. The schematic of the core is shown in Figure 5.

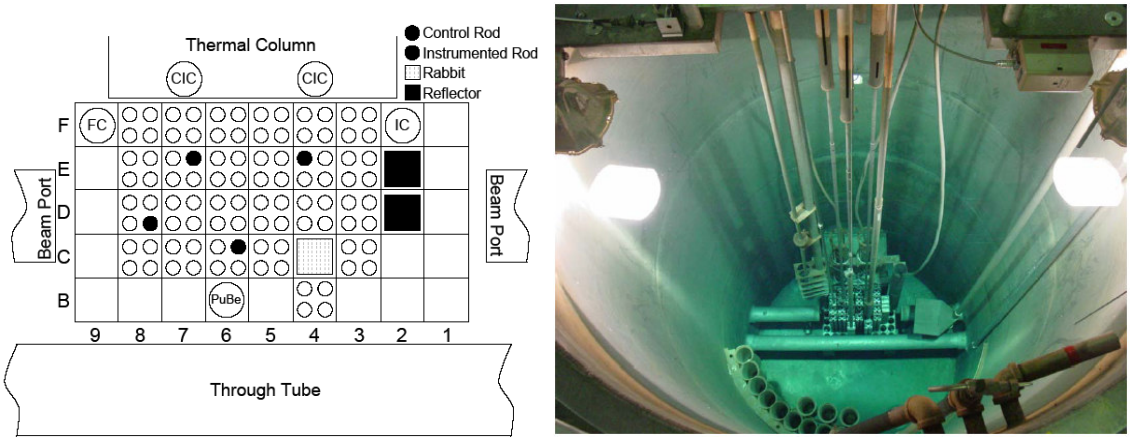


**FIGURE 5.** MUTR core.

The thermal column (TC) experimental facility will be utilized for the *in vitro* irradiations. The TC is a graphite filled housing extending from within the back face of the core through the pool tank wall and the concrete shield. The core end of the housing is a 0.953 cm (0.375 in) thick aluminum sleeve that passes through an aluminum nozzle welded to the pool tank wall. The liner extends through the concrete shield and accommodates a steel-concrete shield plug at its outer end. The graphite assembly consists of 25.81 $cm^2$ (4 $in^2$) graphite stringers arranged to form a stepped column 1.52 m (5 ft) long. The section forward of the step is 0.959 m (37.75 in) long and 0.610 m (2 ft) wide in which the stringers are arranged in a 6 x 6 pattern. The outer section of the graphite column is 0.565 m (22.25 in) long and 0.813 m (32 in) wide and its stringers are arranged in an 8 x 8 pattern

surrounded with 0.318 cm (0.125 in) thick boral. Lead bricks surround the outer end of the TC graphite for shielding of gamma rays.

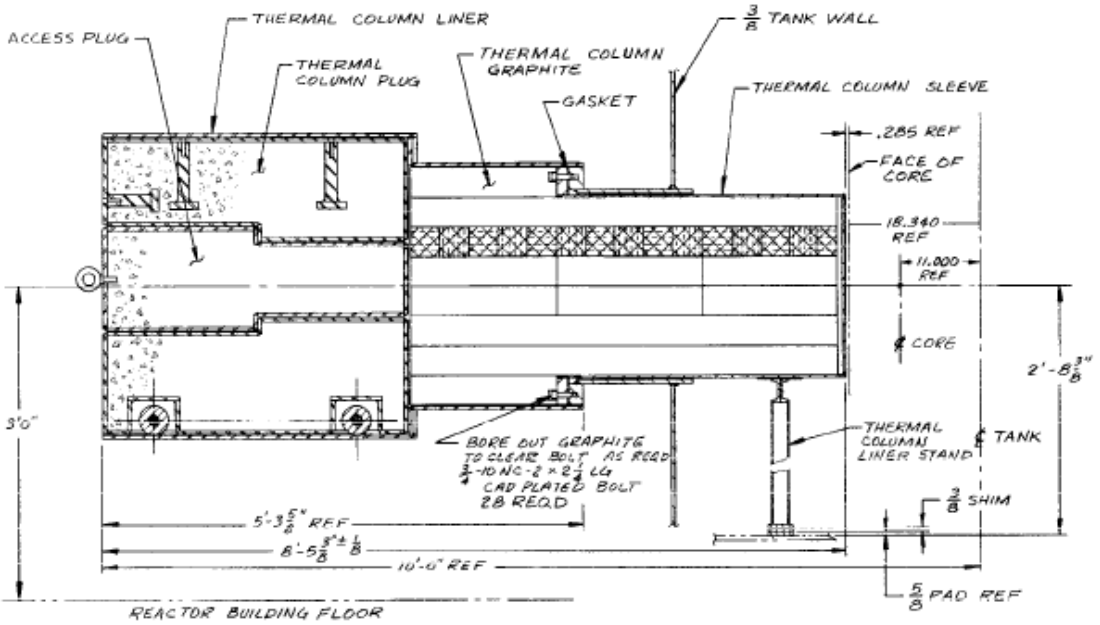


**FIGURE 6.** MUTR thermal column experimental facility.

Thermal neutron flux measurements must be made when operating at various power levels to determine the optimal conditions for irradiation. Current facilities target a flux level higher than $10^8$ n/cm$^2$ in order to keep irradiation times short.

## IV. Research Design and Methods

*Cell Culture*

Human normal prostate RWPE-1 cells (ATCC, Manassas, VA) are grown in Keratinocyte Serum Free Media supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA) at 37$^{o}$C in 5% $CO_2$ in air, and in 75 $cm^2$ tissue culture flasks (Thermo Fisher Scientific). Human cancerous prostate PC-3 cells (ATCC, Manassas, VA) are grown in Ham's F12 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum at 37$^{o}$C in 5% $CO_2$ in air, and in 75 $cm^2$ tissue culture flasks (Thermo Fisher Scientific).

*Preparation and Analysis of Liposomal Formulation*

Liposomes are prepared using DL-α-dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL) (Sigma Aldrich). DPPC and CHOL are added to a 5 mL round bottom flask in a molar ratio of 0.75:0.25, respectively. Specifically, 56.9 mg DPPC and 10 mg CHOL dissolved in chloroform are added. The lipids are then dissolved in 3 mL of a 2:1 (v/v) chloroform-methanol mixture in the round bottom flask. BCH is then added to the mixture in concentration of 10, 15, and 20 mM. A heat gun is used to initiate evaporation of the solvents while turning the flask so that a thin layer of dried lipids is formed on the flask wall. Following this initial drying, the flask is placed in a vacuum desiccator for at least 12 hours. The flask is then removed from the vacuum and the dried lipids are hydrated with 5 mL of preheated PBS on a stirring hot plate for 30 min. Throughout the hydration process, the mixture is kept above 50$^{o}$C to avoid falling below the phase transition temperature ($T_m$) of the lipids. Once hydrated, the MLVs are allowed to mature for 1 hour at room temperature. Size reduction of the MLVs is performed by passing the hydrated solution at least 10 times through an Avanti Mini-Extruder with 50 and 80 nm extrusion membranes in 250 μL syringes (Avanti Polar

Lipids, Alabaster, AL) heated on a block to above 50°C. Each 250 µL sample is collected separately and taken for size analysis.


**FIGURE 7.** Drying and extrusion of liposomal formulation (Avanti Polar Lipids).

Liposome samples are then take to the National Institute of Standards and Technology (NIST) for light scattering analysis using DAWN EOS multi-angle light scattering (MALS), Eclipse 2 Separation System particle separation, and ASTRA software (Wyatt Technology, Santa Barbara, CA). Liposome solutions are diluted to 50µg/100µL in PBS buffer with 200 ppm $NaN_3$. Samples are then placed in vials and inserted into the sample tray. The following settings are used for the samples:

| **Elution flow rate** | **Injection volume** | **Cross flow rate** | **Spacer** | **Elution time following cross flow** | **Total time** |
|---|---|---|---|---|---|
| 1 mL/min | 100 µL | 0.5 to 0 mL/min in 30 min. | 350 µm | 15 min. | 63 min. |

**TABLE 3.** Settings used for AFFF separation.

The sample is then separated by size using asymmetric field flow fractionation (AFFF). Separation is achieved as the sample flows through a chamber that is exposed to a perpendicular cross flow as shown below.

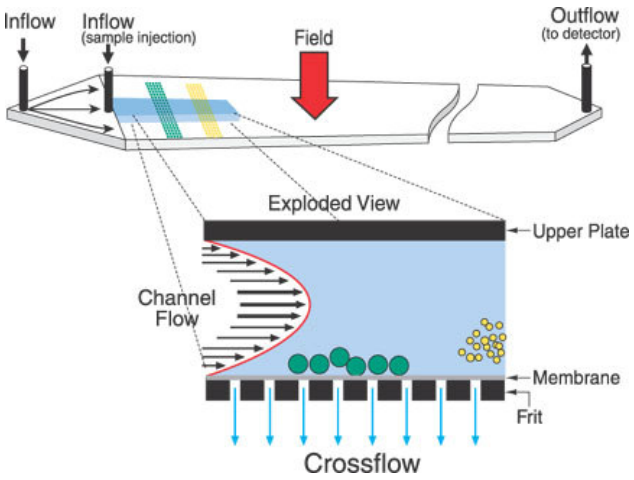


**FIGURE 8.** AFFF size separation (Wyatt Technology).

Within the channel a parabolic flow profile is created that pushes particles closer to the walls of the chamber at a slower rate than those found in the center of the channel. The introduction of a downward cross flow causes the larger particles to move closer to the lower chamber wall, thus positioning them in a slower flow than smaller particles that experience less drag from the cross flow. As the particles move along the channel, smaller particles reach the outflow faster and are sent to the detector first.

As the MLVs pass through the 690 nm beam, the light is scattered and its angular dependence is monitored by eighteen detectors placed around the point of scattering. Because the angular variation of the scattered light is directly related to the size of the molecule, the root mean square radius of the MLVs can be determined using ASTRA software that collects the voltages from each detector. From this, given information about the conformation of the scattering particle, the geometric radius of the sample is calculated. This all occurs according to the basic light scattering equation (Rayleigh-Gans-Debye):

$$I_{scattered}(\theta) \propto R(\theta) = K^{*}McP(\theta)[1-2A_{2}McP(\theta)] \qquad (4)$$

where R(θ) is the excess Raleigh ratio (proportional to intensity of scattered light in excess of light scattered by solvent), M is the molar mass, c is the solute concentration, P(θ) is the form factor, $A_2$ is the second virial coefficient, and $K^*$ is the constant

$(4\pi^2 n_o^2/N_A\lambda_o^4)(dn/dc)^2$ with $n_o$ the solvent refractive index, $N_A$ Avogadro's number, $\lambda_o$ the vacuum wavelength of incident light, and *dn/dc* the specific refractive index increment.

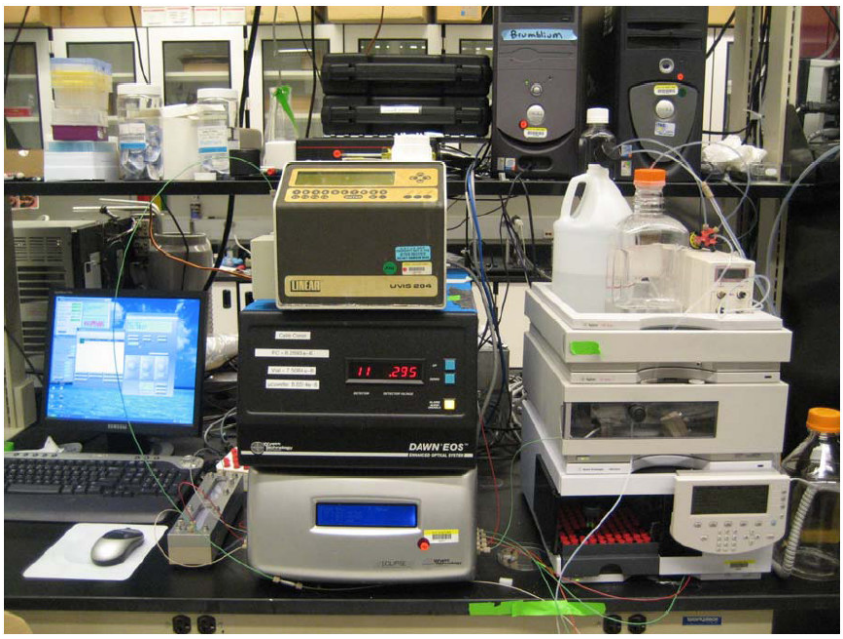


**FIGURE 9.** AFFF and MALS setup.

MLVs were prepared and analyzed by light scattering as described. The Figure below shows the geometric radius of particles passing through the 690 nm beam as a function of time.

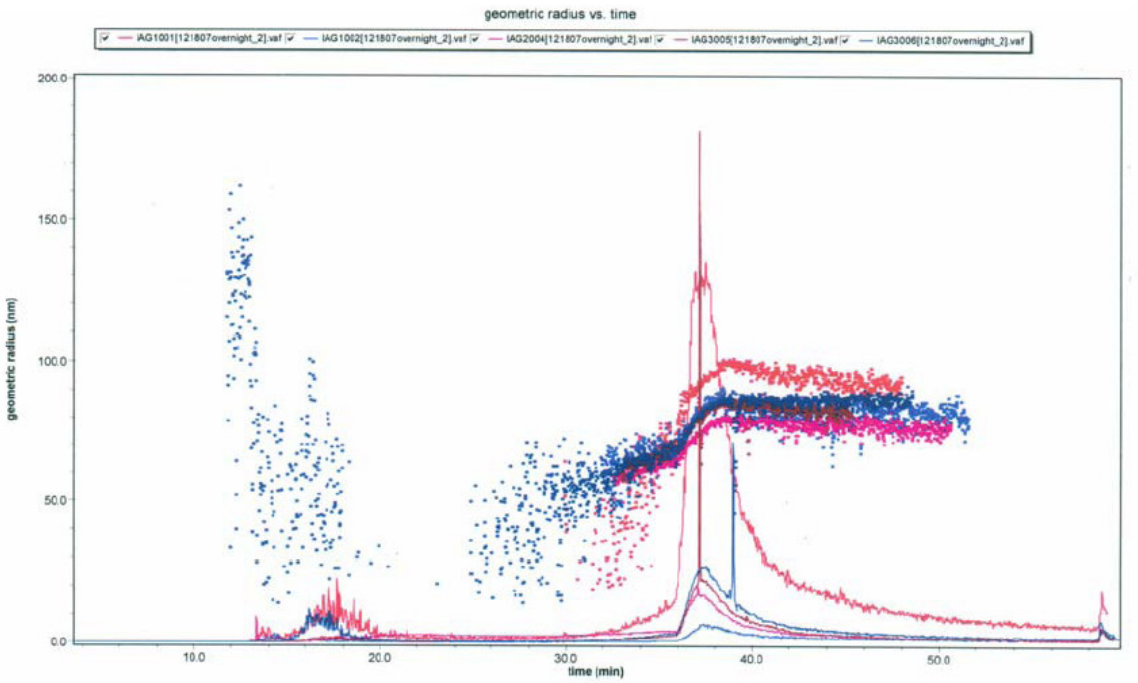


**FIGURE 10.** Geometric radii of liposomes passed through 50 and 80 nm membranes.

The MLVs extruded through the 50 nm membrane appear to have geometric radii between 70-85 nm, while the MLVs extruded through the 80 nm membrane are in the 80-100 nm range. Particles are slightly larger than the membrane through which they are extruded due to swelling that occurs as the particles emerge from the membrane. In order to determine the size of the particles from MALS, the ASTRA software uses the coated sphere model with the reported *dn/dc* value of 0.134 for liposomes, the real refractive index of 1.33 for the PBS solution within the liposomes, and a coating thickness of 9 nm correlating to the size of the lipids surrounding the particle.

*Cellular Uptake and Release of BCH*

All cells will be cultured to semiconfluency and 1 mL of the BCH liposomal formulation will be added to the culture flasks. The concentration of BCH in the liposomal formulation will be 0, 50, 100, and 150 µg/mL. Cells will be cultured with the added BCH liposomal formulation for 2, 16, and 48 hours at 37$^{o}$C. Following this incubation, cells will be washed three times with 0.01 M D-PBSA, pH 7.4 and

disaggregated with tryspin-EDTA. The BCH retention study will be carried out by aspirating the culture medium, replacing it with fresh medium and culturing the cells for an additional 24 hours, followed by washing and harvesting the cells as described above.

Cellular concentrations of the BCH will be determined using high performance liquid chromatography (HPLC). Once the cells are harvested, BCH in the cells will be extracted by a 1:1 (v/v) methanol-isopropanol mixture. A Zorbax Stable Bond C-18 column coupled to an Agilent Tech Series 1100/1200 pump and Linear UVIS 204 system will be used to analyze the BCH in the cells. The column is equilibrated with the mobile phase consisting of 50% methanol and 50% isopropanol at a flow rate of 0.5 mL/min. A standard solution containing 1 mg of cholesterol, BCH, triolein, cholesteryl oleate, and cholesteryl heptadecanoate dissolved in 50 mL of mobile phase solvent will be used as calibration. The UV absorption at the $\lambda_{max}$ of BCH (220nm) will be detected and used to determine the amount of BCH in each cell sample.

*Thermal Column Insert Design, Fabrication, and Characterization*

The thermal column (TC) experimental facility will be used to irradiate the cells, following adaptations made to the original TC insert plug to accommodate biological irradiations. The original TC insert plug is made of solid concrete and is being replaced with an insert plug containing a chamber capable of controlling the environmental conditions of the sample. Environmental control of the sample chamber will be provided by a water jacket and $CO_2$ gas lines connected to external sources. Figure 11 shows a schematic of the modified TC insert plug.

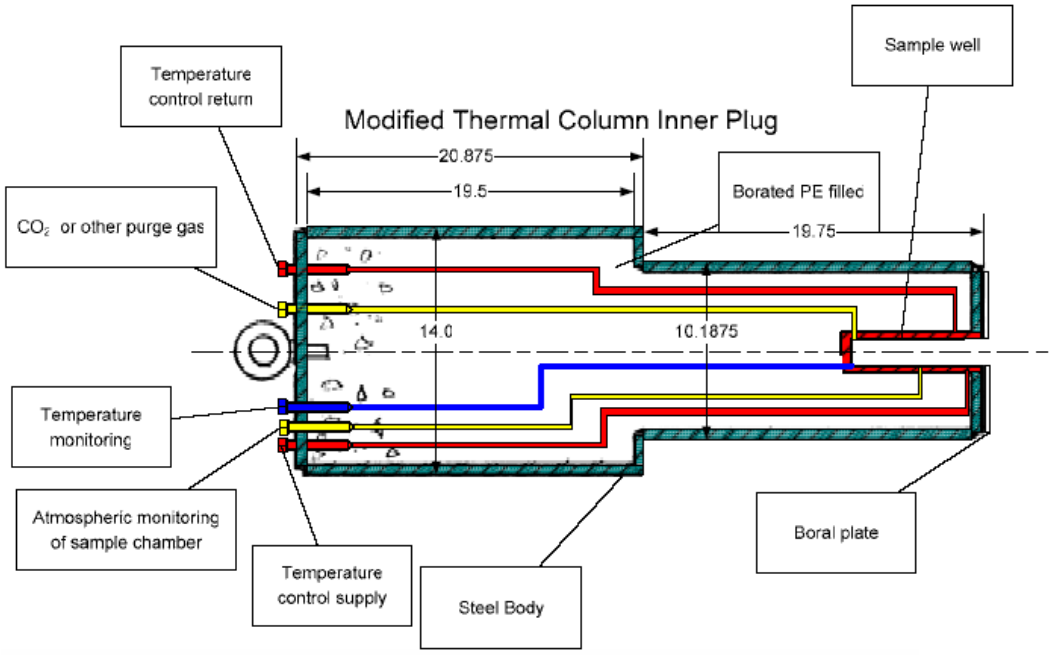


**FIGURE 11.** Schematic of the new TC insert plug.

A $CO_2$ detector and thermocouple embedded in the sample chamber will provide real-time values for the $CO_2$ concentration and temperature. The system will be allowed to equilibrate once the cells have been placed in the chamber, prior to irradiation. This will allow prolonged irradiations which would otherwise result in non-specific cell death. In addition to this, irradiations will be performed at the optimal growing conditions of the cells and will better simulate the physiological response of the cells *in vivo*.

Once the fabrication of the new TC insert plug is complete, beam characterization within the sample chamber will be performed. This will be accomplished through the use of paired ion chambers, foil activation, and a tissue equivalent proportional counter (TEPC). Measurements will be taken at various power levels to determine the optimum conditions for high thermal neutron flux with minimal contribution from gamma rays.

The dual ion chamber technique will be performed with two IC-18, 0.1 cc ion chambers (Far West Technology) used in tandem to detect the individual gamma ray and neutron contributions to the total dose. The first chamber, IC-18G, is made of a 0.064 in. graphite wall filled with air that is sensitive to gamma rays. The second chamber, IC-18, is made of a 0.064 in. tissue equivalent plastic filled with a gas mixture that approximates

the composition of biological tissue (64.4% $CH_4$, 32.4% $CO_2$, 3.2% $N_2$, Airgas) and is sensitive to both gamma rays and neutrons. Ion chambers are not ideal for detection of thermal neutrons and will be better served to give an approximation of the contribution of epithermal and fast neutrons.

In a similar method as described for the IC-18 ion chamber, a SW5 TEPC (Far West Technology) will be used to determine the absorbed dose rate at various power levels. The same tissue equivalent gas mixture will be used to fill the chamber throughout the irradiation. A code adapted from the Pacific Northwest National Laboratories (PNNL) will be used to convert the data into absorbed dose.

In an effort to further unfold the neutron energy spectrum, foil and wire activation will be performed. Foil activation is an indirect detection method that involves inducing radioactivity of a particular material by neutron irradiation. Exposing a foil sample to a neutron flux for a specific period of time, followed by counting with a high purity germanium detector (HPGe), the number and energy distribution of the neutrons can be determined. The rate of this activation is determined by:

$$R = A_\infty = \phi \Sigma_{act} V \qquad (5)$$

In the above formula, R is the rate of activation, $A_\infty$ is the saturated activity, $\phi$ is the neutron flux, $\Sigma_{act}$ is the activation cross section, and V is the foil volume. As the foil is irradiated, the activity A increases as a function of time according to the equation:

$$A_o = A_\infty(1-e^{-\lambda to}) \qquad (6)$$

where $\lambda$ is the decay constant and t is time. It is important to note that the activity of the foil will decrease immediately after removal from the neutron beam and therefore foil counting must be performed quickly and the time delay must be accounted for. The following figure shows the activity of a sample throughout this process.

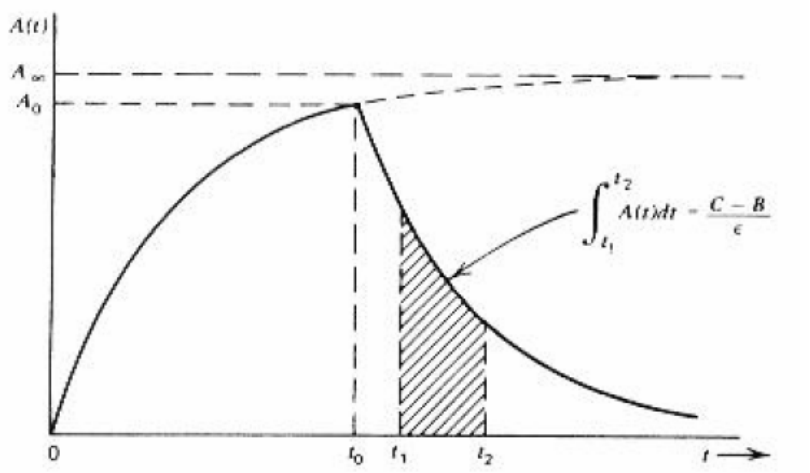


**FIGURE 12.** The activity of a foil throughout and following irradiation.

The counting C is determined as follows:

$$C = \varepsilon \int_{t1}^{t2} A_o e^{-\lambda(t-to)} dt + B = \varepsilon \frac{A_o}{\lambda} e^{\lambda to} (e^{-\lambda t1} - e^{-\lambda t2}) + B \qquad (7)$$

where ε is the counting efficiency and B is the number of background counts. By combining equations 6 and 7 the saturated activity can be determined from equation 8, leading to the neutron flux magnitude from equation 5.

$$A_\infty = \frac{\lambda(C-B)}{\varepsilon(1-e^{-\lambda to})e^{\lambda to}(e^{-\lambda t1} - e^{-\lambda t2})} \qquad (8)$$

The following foils (Alfa Aeser) will be used: W, Ti, Fe, Cu, Ni, Co, Al, V, Mg, Sc, Au, and In. For the threshold activation studies, foils will be irradiated alone and then irradiated again when wrapped in Cd, In, Ag, and depleted U. Gold and indium foils are especially valuable for the determination of the thermal neutron spectrum. The following tables indicate properties of the materials used for activation detection.

| Element | Isotope (Abundance in Percent) | Thermal Activation Microscopic Cross Section (in $10^{-28}$ m$^2$) | Induced Activity | Half-Life |
|---|---|---|---|---|
| Manganese | $^{55}$Mn (100) | 13.2 ± 0.1 | $^{56}$Mn | 2.58 h |
| Cobalt | $^{59}$Co(100) | 16.9 ± 1.5 | $^{60m}$Co | 10.4 min |
| | | 20.2 ± 1.9 | $^{60}$Co | 5.28 y |
| Copper | $^{63}$Cu(69.1) | 4.41 ± 0.20 | $^{64}$Cu | 12.87 h |
| | $^{65}$Cu(30.9) | 1.8 ± 0.4 | $^{66}$Cu | 5.14 min |
| Silver | $^{107}$Ag(51.35) | 45 ± 4 | $^{108}$Ag | 2.3 min |
| | $^{109}$Ag(48.65) | 3.2 ± 0.4 | $^{110m}$Ag | 253 d |
| Indium | $^{113}$In(4.23) | 56 ± 12 | $^{114m1}$In | 49 d |
| | | 2.0 ± 0.6 | $^{114}$In | 72 s |
| | $^{115}$In(95.77) | 160 ± 2 | $^{116m1}$In | 54.12 min |
| | | 42 ± 1 | $^{116}$In | 14.1 s |
| Dysprosium | $^{164}$Dy(28.18) | 2000 ± 200 | $^{165m}$Dy | 1.3 min |
| | | 800 ± 100 | $^{165}$Dy | 140 min |
| Gold | $^{197}$Au (100) | 98.5 ± 0.4 | $^{198}$Au | 2.695 d |

**TABLE 4.** Materials useful as slow-neutron activation detectors.[47]

| Material | Reactions of Interest | Isotopic Abundance (at %) | Half-Life | γ Energy (MeV) | γ Abundance (%) | Threshold (MeV) |
|---|---|---|---|---|---|---|
| F | $^{19}$F(n, 2n)$^{18}$F | 100.0 | 109.7 min | 0.511[+] | 194[o] | 11.6 |
| Mg | $^{24}$Mg(n, p)$^{24}$Na | 78.7 | 15.0 h | 1.368 | 100 | 6.0 |
| Al | $^{27}$Al(n, α)$^{24}$Na | 100.0 | 15.0 h | 1.368 | 100 | 4.9 |
| Al | $^{27}$Al(n, p)$^{27}$Mg | 100.0 | 9.46 min | 0.84–1.01 | 100 | 3.8 |
| Fe | $^{56}$Fe(n, p)$^{56}$Mn | 91.7 | 2.56 h | 0.84 | 99 | 4.9 |
| Co | $^{59}$Co(n, α)$^{56}$Mn | 100.0 | 2.56 h | 0.84 | 99 | 5.2 |
| Ni | $^{58}$Ni(n, 2n)$^{57}$Ni | 67.9 | 36.0 h | 1.37 | 86 | 13.0 |
| Ni | $^{58}$Ni(n, p)$^{58}$Co | 67.9 | 71.6 d | 0.81 | 99 | 1.9 |
| Cu | $^{63}$Cu(n, 2n)$^{62}$Cu | 69.1 | 9.8 min | 0.511[+] | 195[o] | 11.9 |
| Cu | $^{65}$Cu(n, 2n)$^{64}$Cu | 30.9 | 12.7 h | 0.511[+] | 37.8[o] | 11.9 |
| Zn | $^{64}$Zn(n, p)$^{64}$Cu | 48.8 | 12.7 h | 0.511[+] | 37.8[o] | 2.0 |
| In | $^{115}$In(n, n′)$^{115m}$In | 95.7 | 4.50 h | 0.335 | 48 | 0.5 |
| I | $^{127}$I(n, 2n)$^{126}$I | 100.0 | 13.0 d | 0.667 | 33 | 9.3 |
| Au | $^{197}$Au(n, 2n)$^{196}$Au | 100.0 | 6.18 d | 0.33–0.35 | 25–94 | 8.6 |
| Li | $^{7}$Li(n, αn′)t | 92.58 | 12.3 y | 0–0.019[×] | 100[×] | 3.8 |

[+]Annihilation radiation.

[o]Yield of annihilation photons assuming all positrons are stopped.

[×]β particle energy and percent abundance.

**TABLE 5.** Materials useful as threshold activation detectors.[48]

Irradiated samples will then be counted using a GC2020 HPGe detector (Canberra) with Genie 2000 software and applied to the MXD_FC33 code (Physikalisch-Technische Bundesanstalt, Germany) to provide the neutron energy spectrum within the TC.

The results will then be compared to the MCNP-5C model that was previously written for the University of Maryland research reactor facility.[49] Radiation surveying at various power levels and locations within the reactor has been used to benchmark the coded simulation and will be improved as better characterization of the TC experimental facility is completed.

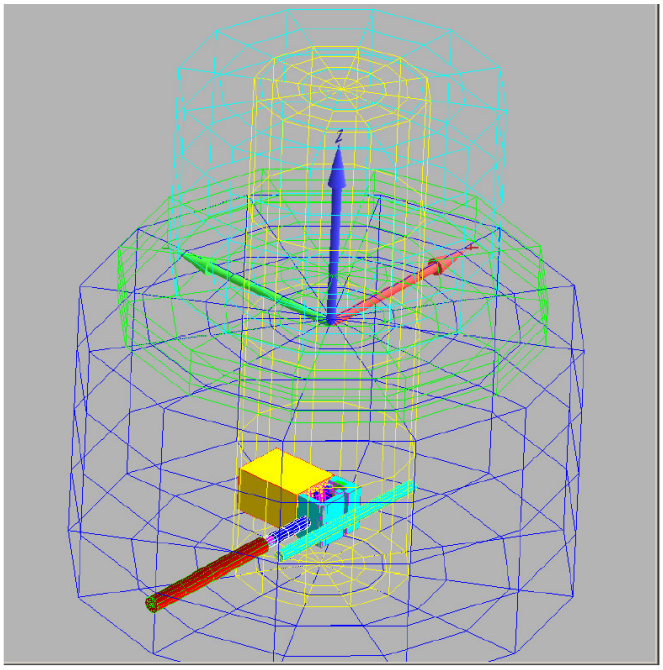

**FIGURE 13**. Simulation of the MUTR with MCNP-5C.

*Thermal Neutron Irradiation*

Prior to irradiation, each tissue flask containing BCH will be washed as described above and filled with fresh media. Tissue flasks containing the RWPE-1 and PC-3 cells will be transferred to an incubator within the MUTR facility. The sample chamber within the TC insert plug will be set to 37°C and 5% $CO_2$ throughout the irradiation. Each flask will be irradiated separately, with matching irradiations performed for normal and cancer

cells. Cells will be irradiated to 0, 2, 4, 6, and 8 Gy, performed in triplicate for each dose and cell type. Immediately following irradiation, cells will be removed from the TC insert plug and transferred to the incubator for the cell survival assay.

*Cell Survival Assay*

Following irradiation, media will be aspirated from the tissue flasks and the cells will be washed with 8 mL of D-PBSA. The cells will be detached from the tissue flasks by addition of trypsin-EDTA and the cell suspension will then be centrifuged at 125 *g* for 6 min. Cell pellets will be resuspended in fresh media and cell density will be counted using a hemocytometer. Cells will then be diluted to the appropriate cell densities for plating. Ideally, there should be approximately 50 surviving cells per 25 $cm^2$ tissue flask. Therefore, depending on the plating efficiency of each cell line and the dose delivered to the sample, the number of cells plated must be adjusted accordingly. The cells are then incubated at 37$^{o}$C and 5% $CO_2$ for 10-14 days. This will allow the colonies to grow large enough to be seen with the naked eye once stained. After 10-14 days, the media will be aspirated from the cells and the cells will be washed with 8 mL of D-PBSA. A mixture of 5 mL D-PBSA and 5 mL methanol is then used to wash the cells, followed by cell fixing with 10 mL of methanol for 10 min. After aspirating the methanol, 5 mL of 0.1% crystal violet solution is added to the cells for 10 min. The stain solution is then aspirated and the cells are washed with $H_2O$ and dried. Once dried, the surviving colonies are counted.

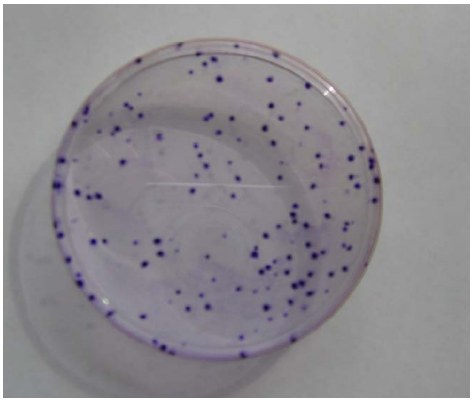

**FIGURE 14.** Staining of surviving colonies with crystal violet.

When performing the cell survival assay there are two important factors that must be accounted for. First, the plating efficiency of the cell lines must be determined. This is accomplished by following the same procedure outlined above, but without any irradiation. By simply detaching the cells from one tissue flask and re-plating them in another, the viability of cells subjected to such a transfer can be determined. The plating efficiency is used to help determine the number of cells to be plated following irradiation and is subtracted out from the overall cell death to isolate cell death that is attributed to radiation alone. Second, the cell death due to gamma ray contribution in the mixed LET irradiation must also be accounted for. To accomplish this, dosimetry will be used to identify the gamma ray dose delivered to the cells during irradiation in the TC. Once this has been established for each dose, cell irradiations will be repeated as described above using the 124 kCi $^{60}$Co gamma source.


**FIGURE 15.** $^{60}$Co gamma source.

Cells will be exposed to the liposomal formulation and then transferred to the gamma cell for irradiation. Calibration of the dose rates within the gamma source has been completed with NIST alanine dosimetry, and cells will be irradiated with the same dose rates and total dose experienced in the reactor TC. By conducting cell survival assays from these gamma ray irradiations, the mixed LET reactor irradiation can be separated into cell death from gamma rays and thermal neutrons (non-specific and specific cell death).

*Timeline*

Preparation and analysis of the liposomes containing BCH and plating efficiency studies for RWPE-1 and PC-3 cell lines are currently under investigation. Initial studies to measure BCH uptake by the cell lines has begun and will continue for each BCH concentration and incubation time throughout the Spring 2008 semester. Fabrication of the TC insert plug has begun and will be completed by March/April. Immediately following completion, beam characterization within the sample chamber will be performed and completed by the end of April. Cell irradiations within the gamma cell and MUTR will then begin and carry on throughout the Fall 2008 semester. Once the cell survival assays for each dose has been performed, the next few months will be spent submitting final papers and writing my dissertation. I expect to complete this research plan and defend my dissertation in the Spring 2009 semester.

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